| Notice of Allowability | Application No. | Applicant(s) | |
|---|---|---|---|
| | 09/847,946 | MAY ET AL. | |
| | Examiner | Art Unit | |
| | Anand U. Desai, Ph.D. | 1653 | |

***-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--***

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *October 12, 2005*.

2. ☒ The allowed claim(s) is/are *3-13*.

3. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

5. ☐ CORRECTED DRAWINGS ( as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review ( PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   **Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☐ Information Disclosure Statements (PTO-1449 or PTO/SB/08), Paper No./Mail Date _____
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application (PTO-152)
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

**JON WEBER**
**SUPERVISORY PATENT EXAMINER**

## DETAILED ACTION

1. This office action is in response to Amendment filed on October 12, 2005. Claims 3-13 are currently pending and are under examination.

**Withdrawal of Rejections**

**2.** The rejection of claims 3-13 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1, and 5 of U.S. Patent No. 6,864,355 B1 is withdrawn based on the filing of a terminal disclaimer.

## EXAMINER'S AMENDMENT

3. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Dr. Zacharakis on November 7, 2005.

The application has been amended as follows:

**Please replace the Description of the Drawings with the following:**

Description Of The Drawings

*Figure 1* depicts results from experiments indicating that NEMO interacts with the COOH-terminus of IKKβ. (A) GST alone or GST-NEMO were precipitated from bacterial lysates using glutathione-agarose, separated by SDS-PAGE (10%) and the gel was stained with Coomassie blue (left panel). Equal amounts of GST or GST-NEMO were used in subsequent GST pull-down experiments. The scheme depicted in the right panel represents the COOH- and NH2-terminal truncation mutants of IKKβ used to determine the region of NEMO interaction. (B) IKKβ mutants were cloned, expressed by

determine the region of NEMO interaction. (B) IKKβ mutants were cloned, expressed by *in vitro* translation (input; left panel) and used for GST pull-down (right panel). (C) Wild-type IKKβ and IKKβ-(644-756) were *in vitro* translated (left panel) and used for GST pull-down analysis (left panel). (D) HeLa cells were transiently transfected with either vector alone or increasing concentrations (0.25, 0.5, 1.0 μg/ml) of the xpress-tagged IKKβ-(644-756) construct together with the pBIIX-luciferase reporter plasmid. After forty-eight hours cells were treated with either TNFα (10 ng/ml) or IL-1β (10 ng/ml) for four hours then NF-κB activity was measured. Western blot analysis from portions of the lysate using anti-xpress (inset) demonstrates the increasing levels of expressed protein.

*Figure 2* depicts results from experiments indicating that the first α-helical region of NEMO is required for binding to IKKβ. (A) A truncated version of IKKβ consisting of only the COOH-terminus from residue V644 to S756 was fused with GST (GST-644-756) and expressed in bacteria. After precipitation by glutathione agarose, GST alone and GST-(644-756) were separated by SDS-PAGE (10%) and the gel was stained with Coomassie blue (left panel). Equal amounts of each protein were used for subsequent GST pull-down analyses. Various NH2- and COOH-terminal truncations of NEMO were constructed, [$^{35}$S]-methionine labeled and used for *in vitro* pull down (right panel). Mutants that interacted with GST-(644-756) are indicated (+). None of the mutants interacted with GST alone. (B) Wild-type NEMO and a deletion mutant lacking the first α-helical region (del.αH) were *in vitro* translated (left panel: input) and used for GST pull-down using the proteins shown above (A: left). (C) HeLa cells were transfected with pBIIx-luciferase together with either pcDNA-3 (vector) or increasing concentrations of del.αH (0.25, 0.5, 1.0 μg/ml) for forty-eight hours then treated for four hours with TNFα (10 ng/ml). Cells were then lysed and NF-κB activity was measured by luciferase assay.

*Figure 3* depicts results from experiments indicating that interaction with NEMO and functional kinase activity requires an IKKα-homologous region of the IKKβ COOH-terminus. (A) Truncation mutations of IKKβ sequentially omitting the extreme COOH-terminus (1-733), the serine-free region (1-707), the serine rich-domain (1-662) and the α1-region (1-644) were expressed and labeled by *in vitro* translation and used for GST pull-down by GST-NEMO (Figure 1A). None of the mutants interacted with GST alone. (B) Sequence alignment of the extreme COOH-termini of IKKβ and IKKα. The α2- and glutamate-rich regions are indicated and the six identical amino acids are shaded. (C) Wild-type IKKβ and the truncation mutants (1-733 and 1-744) were [$^{35}$S]-methionine-labeled (input) and used for *in vitro* pull down with either GST alone or GST-NEMO. (D) ~~HeLa cells were transfected for forty-eight hours with 1 μg/well of the indicated FLAG-tagged constructs followed by immunoprecipitation using anti-FLAG. The immunoprecipitates were incubated in kinase buffer containing [$^{32}$P]-labeled γATP for fifteen minutes at 30°C then washed with lysis buffer containing 1% Triton-100. Resulting complexes were separated by SDS-PAGE (10%) and visualized by autoradiography (upper panel). The lower panel is an immunoblot from identical samples demonstrating equivalent amounts of transfected protein in each lane. (E)~~ HeLa cells were transfected for 48 hours with 1 μg/ml of the indicated constructs or empty vector (pcDNA-3) together with pBIIx-luciferase. NF-κB activity was determined by luciferase assay. ~~(F) HeLa cells transfected for forty-eight hours with FLAG-tagged versions of either IKKβ (wild-type) or IKKβ-(1-733) were either untreated (-) or treated for seven minutes (+) with TNFα (10 ng/ml). Following lysis and immunoprecipitation using anti-FLAG, immune-complex kinase assay (upper panels) was performed. Identical samples were immunoprecipitated and immunoblotted with anti-FLAG (lower panels).~~

*Figure 4* depicts results from experiments indicating that association of NEMO with IKKβ and IKKα reveals the NEMO binding domain (NBD) to be six COOH-terminal amino acids. (A) COS cells transiently transfected with vector alone, FLAG-tagged IKKα or IKKβ (1 μg/well) or xpress-tagged NEMO (1 μg/well) to a total DNA concentration of 2 μg/well as indicated. Following lysis, immunoprecipitations (IP) were performed using anti-FLAG (M2) and the contents of precipitates visualized by immunoblotting (IB) with either anti-FLAG (M2) or anti-xpress. A portion of pre-IP lysate was immunoblotted with anti-xpress to ensure equivalent levels of NEMO expression in transfected cells. (B) NEMO interacted equally well with both IKKβ and IKKα. (C) Wild-type IKKα and IKKα-(1-737) were expressed and labeled (input) and used for GST pull-down using GST or GST-NEMO. (~~C~~ D) Full length cDNA encoding human IKK*i* was obtained by RT-PCR from HeLa cell mRNA using the Expand™ Long Template PCR System (Boehringer Mannheim), the forward primer (5'-CTAGTCGAATTCACCATGCAGAGCACAGCCAATTAC) (SEQ ID NO: 22) and the reverse primer (3'-CTAGTCTCTAGATTAGACATCAGGAGGTGCTGG) (SEQ ID NO: 23) and cloned into the *EcoR*I and *Xba*I sites of pcDNA-3. GST pull-down analysis was performed using [$^{35}$S]-methionine-labeled IKKα, IKKβ and IKK*i*. (~~D~~ E) A deletion mutant of IKKβ lacking the NBD (del.NBD) was [$^{35}$S]-methionine-labeled (input) and used for GST pull down analysis. (~~E~~ F) A Fauchere-Pliska hydrophobicity plot of the COOH-terminus (N721-S756) of human IKKβ was generated using MacVector™ (version 6.5.3) software. The NBD (L737-L742) is boxed. (~~F~~ G) COS cells were transfected for forty-eight hours with a total of 2 μg DNA/well of either vector alone, vector plus NEMO-FLAG or NEMO-FLAG plus xpress-tagged versions of IKKβ-(1-744) containing point mutations within the NBD as indicated. Following lysis and

immunoprecipitation using anti-FLAG (M2), immunoblot analysis was performed with either anti-FLAG or anti-xpress. The level of expressed protein in pre-IP lysate was determined by immunoblotting with anti-xpress (lower panel). (~~G~~ H) HeLa cells were transiently transfected for forty-eight hours with the indicated constructs together with pBIIX-luciferase and NFκB activity in lysate was measured by luciferase assay.

*Figure 5* depicts results from experiments indicating that a cell-permeable peptide spanning the IKKβ NBD inhibits the IKKβ/NEMO interaction, TNFα-induced NF-κB activation and NF-κB-dependent gene expression. (A) Sequences of wild-type and mutant forms of IKKβ NBD peptide. (B) GST-pull-down analysis was performed using either GST-NEMO-*in vitro* translated IKKβ (upper panel) or GST-IKKβ-(644-756)-*in vitro* translated NEMO (lower panel). The assay was performed in the absence (no peptide) or presence of increasing concentrations (125, 250, 500 or 1000 μM) of either mutant (MUT) or wild-type (WT) NBD peptide. (~~B~~ C) HeLa cells were incubated with either peptide (200 μM) for the times indicated. Following lysis, the IKK complex was immunoprecipitated using anti-NEMO and the resulting immunoblot probed with anti-IKKβ. (~~C~~ D) ~~HeLa cells were transfected for forty-eight hours with pBIIX-luciferase then incubated for two hours in the absence (control) or presence of mutant or wild-type NBD peptide (100 and 200 μM of each). Subsequently the cells were either treated with TNFα (10 ng/ml) as indicated (left panel) or left untreated (right panel) for a further four hours after which NF-κB activation was measured by luciferase assay.~~ Gel image showing anti-NEMO immunoprecipitation. (E) Gel image showing anti-FLAG immunoblot. (~~D~~ F) HeLa cells were incubated for three hours with increasing concentrations (50, 100 or 200 μM) of each peptide followed by treatment for fifteen minutes with TNFα (10 ng/ml) as indicated (+). Following lysis, nuclear extracts were

made and 10 μg of protein from each sample was used for EMSA using a specific [$^{32}$P]-labeled κB-site probe. ~~(E) Primary HUVEC were pre-incubated for two hours with of wild-type (left) or mutant (right) NBD peptides (100 μM) then stimulated with TNFα (10 ng/ml) for a further six hours. Control cells received no peptide. Cells were stained with either anti-E-selectin (H4/18) or a non-binding control antibody (K16/16) and expression was measured by FACS (FACSort, Becton Dickinson). The profiles show E-selectin staining in the absence (shaded) and presence (solid line) of TNFα and control antibody staining under the same conditions (dashed line, no TNFα; dotted line, TNFα).~~ (G) Gel image showing anti-Phospho-C-Jun immunoblot and anti-β-Actin immunoblot. (H) HeLa cells were transfected for forty-eight hours with pBIIX-luciferase then incubated for two hours in the absence (control) or presence of mutant or wild-type NBD peptide (100 and 200 μM of each). Subsequently the cells were either treated with TNFα (10 ng/ml) as indicated (top panel) or left untreated (bottom panel) for a further four hours after which NF-κB activation was measured by luciferase assay.

*Figure 6* depicts results from experiments indicating that the wild-type NBD peptide inhibits NF-κB-induced gene expression and experimentally induced inflammation. (A) Primary HUVEC were pre-incubated for two hours with wild-type (middle) or mutant (bottom) NBD peptides (100 μM) then stimulated with TNFα (10 ng/ml) for a further six hours. Control cells received no peptide. Cells were stained with either anti-E-selectin (H4/18) or a non-binding control antibody (K16/16) and expression was measured by FACS (FACSort, Becton Dickinson). The profiles show E-selectin staining in the absence (shaded) and presence (solid line) of TNFα and control antibody staining under the same conditions (dashed line, no TNFα; dotted line, TNFα). (B) % control release of $NO_2$- in various samples. (C) PMA-induced ear edema in mice

topically treated with either vehicle (VEH), dexamethasone (DEX) or NBD peptides was induced and measured as described in Example 8. Data are presented as mean differences in ear thickness ± SD (* = $p < 0.05$ compared with both untreated control [-] and vehicle [VEH]). (~~B~~ D) The effects of the NBD peptide compared with the effect of dexamethasone (DEX) on Zymosan (ZYM)-induced peritonitis in mice were determined as described again in Example 8. Control mice were injected with phosphate-buffered saline (PBS).

*Figure 7* depicts results from experiments indicating the dose dependent inhibition of osteoclast differentiation by wild-type but not mutant NBD peptides. Data are presented as the mean determination of triplicate samples ± SD.

*Figure 8* depicts the results of a mutational analysis of D738 within the NEMO binding domain (NBD) of human IKKβ. (A) The aspartic acid residue at position 738 of IKKβ was substituted with either alanine, asparagine or glutamic acid using PCR-mutagenesis. (B) The IKKβ (D738) mutants shown in A were $^{35}$S-methionine-labeled by *in vitro* transcription and translation then used for GST pull-down analysis using GST-NEMO as previously described. (C) Hela cells were transiently transfected using the Fugene6 transfection method with the NF-κB-dependent reporter construct pBIIx-luciferase together with either pcDNA-3, IKKβ or the D738 mutants described above in (A). After 48 hours, the cells were lysed and luciferase activity was determined as previously described.

*Figure 9* depicts the results of a mutational analysis of W739 and W741 within the NBD of human IKKβ. (A) The tryptophan residues at positions 739 and 741 of IKKβ were substituted with alanine, phenylalanine, tyrosine or arginine using PCR-mutagenesis. (B) COS cells were transiently transfected with either vector alone (pcDNA-

3.1-xpress), IKKβ, W739A, W739F or W739Y together with FLAG-tagged NEMO as shown. After 48 hours, the cells were lysed and complexes were immunoprecipitated (IP) using anti-FLAG (M2)-coupled agarose beads. Prior to immunoprecipitation a portion of each lystate (5%) was retained for analysis (pre-IP). Proteins in samples were separated by SDS-PAGE (10%) and analyzed by immunoblotting (IB) using antibodies recognizing either FLAG (M2) or xpress. The upper two panels show xpress-tagged IKKβ and the lower panel shows FLAG-tagged NEMO. (C and D) COS cells were transiently transfected with the plasmids shown followed by immunoprecipitation and immunoblot analysis as described in B. ~~(C and D)~~ (E and F) Hela cells were transiently transfected with pBIIx-luciferase together with the plasmids shown and after 48 hours luciferase activity in lysates was determined.

*Figure 10* depicts the results of a mutational analysis of S740 within the NBD of human IKKβ. (A) The serine residues at position 740 of IKKβ was substituted with alanine or glutamic acid using PCR-mutagenesis. (B) COS cells were transiently transfected with the plasmids shown followed by immunoprecipitation and immunoblot analysis as described in Fig.2B. (C) Hela cells were transiently transfected for 48 hours with either IKKβ-FLAG or S740E-FLAG then treated for the times shown with TNFα (1μg/ml). Following lysis, complexes were precipitated using anti-FLAG (M2)-coupled agarose beads and an immune-complex kinase assay was performed using GST-IκBα (1-90) as a substrate as previously described.

*Figure 11* depicts the results of a mutational analysis of the IKKα NBD. (A) Each of the residues that comprise the NBD of IKKα (L738 to L743) were substituted with alanine by PCR-mutagenesis. COS cells were transiently transfected with NEMO-FLAG together with either vector alone (pcDNA-3.1-xpress) or xpress-tagged versions of IKKα

and the NBD mutants as shown. Immunoprecipitation and immunoblot analysis of the IKKα-NEMO complexes was performed as described in Fig.2B. (B) Hela cells were transiently transfected with pBIIx-luciferase together with the plasmids shown and after 48 hours luciferase activity in lysates was determined.

*Figure 12* depicts the results of an experiment demonstrating that a peptide encompassing the IKKβ NBD prevents the interaction of IKKα with NEMO. (A) Sequences of the NBD wild type and scrambled control peptides. The wild type peptide corresponds to residues 734 to 744 of IKKβ. (B) GST pull-down analysis was performed using GST-NEMO and in vitro transcribed and translated IKKα (upper panel) and IKKβ (middle panel) in the presence or absence of either vehicle (2% DMSO), scrambled or wild type NBD peptide (500 and 1000μM of each peptide). The lower panel shows a coomassie blue-stained gel demonstrating that neither peptide affects the interaction of GST-NEMO with the glutathione-agarose beads used for precipitation.(C) Densitometric analysis of autoradiograph bands obtained following GST pull-down of IKKa and IKKb using GST-NEMO in the presence of a range of concentrations of wild type NBD peptide. The inset shows a representative experiment. The data are presented as the pixel density as a percentage of control (no peptide) and represent means ± sd (n = 11). Analysis was performed using the NIH-Image software.

**Please replace at page 43, line 24 through page 47, line 14 with the following:**

HeLa cells were transfected for forty-eight hours with 1 μg/well of the indicated FLAG-tagged constructs followed by immunoprecipitation using anti-FLAG. The immunoprecipitates were incubated in kinase buffer containing [$^{32}$P]-labeled γATP for fifteen minutes at 30□C then washed with lysis buffer containing 1% Triton-100. Resulting complexes were separated by SDS-PAGE (10%) and visualized by

autoradiography. An immunoblot from identical samples demonstrated equivalent amounts of transfected protein in each lane. HeLa cells transfected for forty-eight hours with FLAG-tagged versions of either IKKβ (wild-type) or IKKβ-(1-733) were also either untreated (-) or treated for seven minutes (+) with TNFα (10 ng/ml). Following lysis and immunoprecipitation using anti-FLAG, immune-complex kinase assay was performed. Identical samples were immunoprecipitated and immunoblotted with anti-FLAG.

IKKβ COOH-terminal truncation mutants were next used to test the effects of NEMO association on basal and induced activity of IKKβ. ~~Figure 3D shows that truncation~~ Truncation of IKKβ at V644, eliminating the serine-rich region (see Figure 3A), resulted in complete loss of basal auto-phosphorylation. In contrast, a mutant containing the serine-rich region (1-733), exhibited significantly higher levels of auto-phosphorylation than wild-type IKKβ ~~(Figure 3D)~~. Intriguingly, the level of auto-phosphorylation of IKKβ-(1-744) which contains the NEMO-binding α2-region, was identical to that observed with the wild-type kinase. To test the effects that these mutations have on basal kinase activity, mutants were transiently transfected into HeLa cells and NF-κB activity determined by luciferase assay as described in Example 1. ~~The results in Figure 3D demonstrate that~~ IKKβ-(1-644) did not induce NF-κB activity whereas IKKβ-(1-733) caused increased activation compared with wild-type (Figure ~~3E~~ 3D). Furthermore, NF-κB activity induced by IKKβ-(1-744) was identical to that induced by wild-type IKKβ. These results demonstrate that basal auto-phosphorylation and kinase activity of IKKβ is dependent on the ability of NEMO to associate with the kinase. One explanation for these observations may be that NEMO recruits a phosphatase to the IKK-complex that regulates basal IKKβ function by targeting the serine-rich region of the

COOH-terminus. Inability to bind NEMO therefore prevents phosphatase recruitment and causes increased phosphorylation within this region.

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To directly test the effect that loss of the α2-region has on the catalytic activity of IKKβ, an immune-complex kinase assay was performed on lysate from transfected HeLa cells (~~Figure 3F~~). For immune-complex kinases assays, precipitates were washed with TNT then with kinase buffer (20 mM HEPES pH 7.5, 20 mM MgCl2, 1 mM EDTA, 2 mM NaF, 2 mM β-glycerophosphate, 1 mM DTT, 10 μM ATP). Precipitates were then incubated for fifteen minutes at 30□C in 20 μl of kinase buffer containing GST-IκBα-(1-90) and 10 μCi [$^{32}$P]-γ-labeled ATP (Amersham-Pharmacia). The substrate was precipitated using glutathione-agarose (Amersham-Pharmacia) and separated by SDS-PAGE (10%). Kinase activity was determined by autoradiography. Phosphorylated proteins associated with the kinase complex appeared on autoradiographs because the immuno-precipitated complex was not removed prior to GST- substrate precipitation.

Activity of IKKβ (wild-type) was low in untreated cells but was markedly enhanced after treatment with TNFα. Consistent with the data presented in Figure ~~3E~~ 3D, basal activity of IKKβ-(1-733) was significantly higher than wild-type, however this activity was not further enhanced by treatment with TNFα ~~(Figure 3F)~~. Furthermore, basal and TNFα-induced catalytic activity of IKKβ-(1-744) was identical to the activity of IKKβ (WT). In addition to phosphorylated GST-IκBα, auto-phosphorylated IKKβ proteins were also detected ~~(Figure 3F, top bands)~~. Following TNFα treatment, IKKβ (WT) and IKKβ-(1-744) became rapidly autophosphorylated whereas the already high basal phosphorylation of IKKβ-(1-733) was only slightly enhanced ~~(Figure 3F)~~. A previous study showed that auto-phosphorylation serves to down-regulate TNFα-induced IKKβ activity by causing conformational changes within the protein (Delhase *et al.*, (1999) Science 284, 309-313). Taken together, these findings (~~Figures 3D-F~~ Figure 3D) demonstrate that in the absence of NEMO, IKKβ becomes auto-phoshorylated, basally active and refractory to TNFα-induced signals indicating that NEMO plays a fundamental role in the down-regulation as well as activation of IKKβ activity.

An additional band representing a phosphorylated protein appeared only in the samples from TNFα-induced IKKβ (WT) and IKKβ-(1-744) transfected cells ~~(Figure 3F)~~. The molecular weight of this protein (49 kDa) strongly suggests that it is endogenous NEMO associated with the precipitated complex. This is supported by the absence of the band in either precipitate (+/- TNFα) from IKKβ-(1-733) transfected cells. This protein has been identified as phosphorylated NEMO by dissociating the precipitated complex in SDS and re-immunoprecipitating [$^{32}$P]-labeled NEMO using specific anti-NEMO antibodies. Induced phosphorylation of NEMO may therefore represent a further level of regulation of the activity of the IKK complex.

**EXAMPLE 3: IDENTIFICATION OF THE NBD ON IKKA**

Since the α2-region of IKKβ strongly resembles the COOH-terminus of IKKα (Figure 3B), the ability of IKKα to interact with NEMO was tested.

Immunoprecipitations from lysate of COS cells transiently transfected with xpress-tagged NEMO together with FLAG-tagged versions of either IKKα or IKKβ were performed using anti-FLAG as described in Example 1. Figures 4A and 4B show ~~shows~~ that NEMO interacted equally well with both IKKβ and IKKα. It is possible that in this experiment the interaction with IKKα is not direct but due instead to the formation of a complex containing endogenous IKKβ, FLAG-IKKα and xpress-NEMO. A GST-pull-down assays was therefore performed using GST-NEMO and [$^{35}$S]-methionine-labeled versions of either wild-type IKKα or a truncated IKKα mutant lacking the eight COOH-terminal amino acids (1-737: Figure ~~4B~~ 4C). In agreement with

the findings presented above (Figure 4A), but in contrast to previous reports (Mercurio *et al.*, (1999) Mol. Cell. Biol. 19, 1526-1538; Yamaoka *et al.*, (1998) Cell 93, 1231-1240; Rothwarf *et al.*, (1998) Nature 395, 297-300), wild-type IKKα interacted with NEMO *in vitro* whereas the truncated mutant did not (Figure ~~4B~~ 4C). These results not only demonstrate that IKKα interacts with NEMO but also shows that it does so via the COOH-terminal region containing the six amino acids shared between IKKα and the α2-region of IKKβ (Figure 3B). Gene-targeting studies have demonstrated profound differences in the activation of IKKα and IKKβ by TNFα (Woronicz et al., (1997) Science 278, 866-869; Zandi *et al.*, (1997) Cell 91, 243-252; Mercurio *et al.*, (1997) Science 278, 860-866; DiDonato *et al.*, (1997) Nature 388, 548-554; Régnier *et al.*, (1997) Cell 90, 373-383).

The present findings indicate that the basis of this difference is not due to differential recruitment of NEMO (Delhase *et al.*, (1999) Science 284, 309-313; Takeda *et al.*, (1999) Science 284, 313-316; Hu *et al.*, (1999) Science 284, 316-20; Li *et al.*, (1999) Science 284, 321-325; Li *et al.*, (1999) J. Exp. Med. 189, 1839-1845; Li *et al.*, (1999) Genes Dev. 13, 1322-1328; Tanaka *et al.*, (1999) Immunity 10, 421-429). Instead the difference most likely lies in the ability of each kinase to integrate NEMO-associated signaling components into an activation response, presumably through differences in the inherent regulatory features of the individual kinases.

Further evidence that this short COOH-terminal sequence constitutes the NEMO-interaction domain of the IKKs was obtained when we tested the ability of the recently described IKK-related kinase IKK*i* (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362) to interact with NEMO. Sequence comparison with IKKα and IKKβ (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362; Woronicz *et al.*, (1997) Science 278, 866-869; Zandi *et al.*, (1997) Cell 91, 243-52; Mercurio *et al.*, (1997) Science 278, 860-866; DiDonato *et al.*, (1997) Nature 388, 548-554; Régnier *et al.*, (1997) Cell 90, 373-383) reveals that IKK*i* does not contain the α2-region in its COOH-terminus (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362) and consistent with this being the NEMO binding domain we found that IKK*i* does not interact with GST-NEMO in pull down assays (Figure ~~4C~~ 4D). This finding indicates that NEMO is not required for the functional activity of IKK*i* and this is supported by the inability of IKK*i* to respond to signals induced by either TNFα or IL-1β (Shimada *et al.*, (1999) Int. Immunol. 11, 1357-1362).

**EXAMPLE 4: MUTATION OF AMINO ACID RESIDUES IN THE NBD**

Having determined that the α2-region of IKKβ, and the equivalent six amino acid sequence of IKKα are critical for interaction with NEMO [designated NEMO binding domain

(NBD)], a deletion mutant in IKKβ lacking the six amino acids from L737 to L742 (del.NBD) was constructed. This deletion mutant did not associate with GST-NEMO (Figure 4D). Examination of predicted structural and biochemical features of the NBD in context with surrounding residues suggests that it constitutes an inflexible hydrophobic "pocket" within a hydrophilic region of the IKKβ COOH-terminus (Figure ~~4E~~ 4F). This suggests a model in which the NBD becomes buried within the first α-helical region of bound NEMO (Figure 2) preventing its exposure to an aqueous environment thereby maintaining a strong inter-molecular interaction. Whether the interaction is indeed a function of this hydrophobicity remains to be determined, however we found that substitution of either W739 or W741 with alanine prevented association of NEMO with IKKβ (Figure ~~4F~~ 4G). Therefore each of these hydrophobic tryptophan residues is critical for maintaining a functional NBD. In addition, mutation of D738 to alanine also prevented NEMO interaction indicating that a negatively charged residue at this position is required for NBD function. In contrast to these mutations, substitution of L737, S740 or L742 with alanine did not affect NEMO binding. To test the effects of these alanine substitutions on IKKβ function, HeLa cells were co-transfected with each of the point mutants together with pBIIX-luciferase reporter. Consistent with the observation that the basal activity of IKKβ is increased in the absence of associated NEMO, IKKβ-(1-733) (Figure ~~3E~~ 3D), mutants that did not bind NEMO (D738A, W739A and W741A) activated NF-κB to a greater extent than wild-type IKKβ or IKKβ-(1-744) (Figure ~~4G~~ 4H). In contrast, mutants containing substitutions that did not disrupt NEMO association (L737A, S740A and L742A) induced NF-κB to the same level as the controls. These results indicate that NEMO plays a critical role in the down-regulation of intrinsic IKKβ activity.

Further mutations in the NBD were analyzed (see Table 1) for their ability to affect NEMO binding to IKKβ using the GST pulldown assay explained in Example 3.

**Please replace the text of Examples 5-8, beginning on page 48 with the following:**

## EXAMPLE 5: AGENTS WHICH INTERACT WITH NBD TO BLOCK NEMO BINDING

The relatively small size of the NBD makes it an attractive target for the development of compounds aimed at disrupting the core IKK complex. The relevance of this approach was investigated by designing cell-permeable peptides spanning the IKKβ NBD and determining their ability to dissociate the IKKβ-NEMO interaction.

The sequences of the two NBD peptides used in this study were [DRQIKIWFQNRRMKWKK]TALDWSWLQTE (wild-type) (SEQ ID NO:18) and [DRQIKIWFQNRRMKWKK]TALDASALQTE (mutant) (SEQ ID NO:19); Figure 5A). The *antennapedia* homeodomain sequence (Derossi *et al.*, (1994) J. Biol. Chem. 269, 10444-10450; U.S. Patent No. 5,888,762; U.S. Patent No. 6,015,787; U.S. Patent No. 6,080,724) is bracketed and the positions of the Wto A mutations are underlined. Both peptides were dissolved in DMSO to a stock concentration of 20 mM. For all experiments DMSO alone controls were no different from no peptide controls.

The wild-type NBD peptide consisted of the region from T735 to E745 of IKKβ fused with a sequence derived from the third helix of the antennapedia homeodomain that has been shown to mediate membrane translocation (Derossi *et al.*, (1994) J. Biol. Chem. 269, 10444-10450). The mutant was identical except that the tryptophan residues (W739 and W741) in the NBD were mutated to alanine. Figure 5A 5B shows that the NBD (WT) but not the mutant dose-dependently inhibited *in vitro* pull-down of [$^{35}$S]-labeled IKKβ by GST-NEMO and [$^{35}$S]-labeled NEMO by GST-IKKβ-(644-756). To test the ability of the NBD peptides to enter cells and inhibit the IKKβ-NEMO interaction, HeLa cells were incubated with the peptides for different time periods and immunoprecipitated the IKK complex using anti-NEMO. In agreement with the *in vitro* data (Figure 5A 5B), wild-type but not mutant disrupted the formation of the endogenous IKK complex (Figure 5B 5C).

**EXAMPLE 6: AGENTS WHICH BLOCK NEMO FUNCTION**

The effects of the NBD peptides on signal-induced activation of NF-κB were investigated next. Analysis using electrophoretic mobility shift assays (EMSA) also demonstrated that only the wild-type NBD peptide inhibited TNFα-induced activation and nuclear translocation of NF-κB (Figure 5F). Further, after ~~After~~ transfecting HeLa cells with the pBIIX-luciferase reporter, cells were preincubated with wild-type or mutant peptides, treated with TNFα and NF-κB activation measured by the luciferase reporter assay. As shown in Figure ~~5C~~ 5H (~~left~~ top panel), the wild-type NBD peptide inhibited TNFα-induced NF-κB activation whereas the mutant had no effect. Interestingly, the basal NF-κB activity was enhanced by treatment with the wild-type peptide (Figure ~~5C~~ 5H; ~~right~~ bottom panel), a finding which concurs with results from previous mutational analysis (Figures ~~3E-F~~ 3D and ~~4G~~ 4H). This indicates that removal of NEMO increases the basal, intrinsic activity of IKK, while abolishing its responsiveness to TNFα. ~~Further analysis using electrophoretic mobility shift assays (EMSA) also demonstrated that only the wild-type NBD peptide inhibited TNFα-induced activation and nuclear translocation of NF-κB (Figure 5D).~~ Taken together these results demonstrate that delivery of an intact NBD peptide into cells disrupts the IKKβ-NEMO interaction and prevents pro-inflammatory signals from activating NF-κB. In contrast, transduction with a peptide containing mutations at the tryptophan residues that are critical for maintaining the NEMO interaction has no effect.

**EXAMPLE 7: AGENTS CAPABLE OF DOWN-REGULATING E-SELECTIN**

Many proteins involved in the initiation and maintenance of inflammatory responses require NF-κB activation for induced expression of their genes (Ghosh *et al.*, (1998) Annu. Rev. Immunol. 16, 225-260; May & Ghosh, (1998) Immunol. Today 19, 80-88). One such protein, E-selectin, is a leukocyte adhesion molecule expressed on the luminal surface of vascular endothelial cells after activation by pro-inflammatory stimuli such as IL-1 or TNFα (Pober *et al.*, (1986) J. Immunol. 436, 1680-1687; Bevilacqua *et al.*, (1987) Proc. Natl. Acad. Sci. USA 84, 9238-9242; Collins *et al.*, (1995) FASEB J. 9, 899-909). Expression of E-selectin and other NF-κB-dependent adhesion molecules is crucial for the arrest and recruitment of leukocytes into sites of acute and chronic inflammation. To assess the anti-inflammatory potential of the NBD peptide, primary human umbilical vein endothelial cells (HUVEC) were pretreated with the wild-type and mutant peptides and E-selectin expression induced with TNFα. Consistent with the effects on basal NF-κB activation (Figure ~~5C~~ 5H), the wild-type NBD peptide induced low level expression of E-selectin (Figure ~~5E~~ 6A). However, after TNFα-treatment the wild-type but

not mutant significantly reduced expression of E-selectin (Figure ~~5E~~ 6A). Inhibition by wild-type NBD peptide reduced expression to the level induced by the peptide in the absence of TNFα.

The importance of the present invention can be viewed on two levels. First, Applicants have identified the structural requirements for the association of NEMO with the IKKs and found that association with IKKβ is dependent on three amino acids (D738, W739 and W741) within the NBD. Furthermore, NEMO not only functions in the activation of IKKβ but it also has a critical role in suppressing the intrinsic, basal activity of the IKK complex. The second level of importance is the obvious clinical use for drugs targeting the NBD. Applicants have demonstrated that a cell-permeable peptide encompassing the NBD is able to not only inhibit TNFα-induced NF-κB activation but also reduce expression of E-selectin, an NF-κB-dependent target gene, in primary human endothelial cells. The NBD is only six amino acids long, and therefore it is within the ability of one skilled in the art to design peptido-mimetic compounds that disrupt the core IKK complex. Since the effect of disrupting the complex is to increase the basal activity of the IKK, treatment with an NBD-targeting compound can avoid issues of toxicity, *e.g.*, due to hepatocyte apoptosis, that might arise from administering drugs that completely abolish the activity of NF-κB. Hence, identification of the NBD is a means for the development of novel anti-inflammatory drugs that prevent activating signals from reaching the IKK complex, yet maintain a low level of NF-κB activity and avoid potential toxic side-effects.

## EXAMPLE 8: NBD PEPTIDE-MEDIATED INHIBITION OF INFLAMMATORY RESPONSE *IN VIVO*

The NBD peptide was tested for its ability to inhibit inflammatory responses in animals using two distinct models of acute inflammation. In the first model, ear edema was induced in mice using phorbol-12-myristate-13-acetate (PMA) and the effects of topical administration of the NBD peptides were measured. Ear edema using PMA was induced in replicate groups of age and sex matched mice as previously described (Chang *et al.*, (1987) Eur. J. Pharmacol. 142, 197-205). Twenty μl of either NBD peptides (200 μg/ear), dexamethasone (40 μg/ear) or vehicle (DMSO:Ethanol; 25:75 v/v) was applied topically to the right ear of mice thirty minutes before and thirty minutes after the application of 20 μl of PMA (5 μg/ear) dissolved in ethanol. Ear swelling was measured six hours after PMA application using a microgauge and expressed as the mean difference in thickness between the treated (right) and untreated (left) ears. Statistical analysis of the data was performed using the students t-test. A value of $p < 0.05$ was considered statistically significant.

Figure ~~6A-~~6C shows that the wild type peptide significantly reduced (77 ± 3% inhibition; $p < 0.05$) PMA-induced ear thickening to the level observed with dexamethosone (82 ± 9% inhibition; $p < 0.05$). In contrast, the effect observed with an equivalent dose of mutant was insignificant ($p = 0.09$). Neither peptide had an effect when administered in the absence of PMA (not shown).

In a second model, peritonitis was induced in mice by intraperitoneal (i.p.) injection of zymosan either alone or in combination with dexamethasone or the NBD peptides. For zymosan-induced peritonitis, measurement of peritoneal exudates and inflammatory cell collections from replicate groups of age and sex matched mice (C57BL/6NCR) were performed as previously described (Getting *et al.*, (1998) Immunology 95, 625-630). Groups of animals were injected concomitantly with one ml zymosan (1 mg/ml) and either dexamethasone (100 mg/ml) or the NBD peptides (200 mg/ml). The concentration of NOX (nitrate plus nitrite) present in the inflammatory exudates was measured using a colorimetric assay kit (Alexis Corporation) according to the manufacturers protocol.

As shown in Figure ~~6B-~~6D zymosan injection caused an accumulation of inflammatory exudate fluids and migration of polymorphonuclear cells (PMN) into the peritoneum of these animals. Treatment of mice with wild type NBD peptide or dexamethasone significantly reduced exudate formation and PMN accumulation whereas the mutant had no effect.

Various *in vivo* studies have demonstrated a role for NO in exudate formation and leukocyte migration into inflammatory sites (Ialenti *et al.*, (1992) Eur. J. Pharmacol. 211, 177-182; Ialenti *et al.*, (1993) Br. J. Pharmacol. 110, 701-706; Iuvone *et al.*, (1998) Br. J. Pharmacol. 123, 1325-1330). Therefore the effects of the NBD peptides on NOX accumulation in the peritoneal exudates of zymosan-treated mice ~~was~~ were investigated. Figure ~~6C-~~6D (lower panel) shows that dexamethasone and wild-type peptide reduced NOX by 86 ± 7% and 66 ± 4% respectively whereas the mutant had no effect. These results are consistent with previous studies demonstrating that reduction of exudate formation and cell accumulation closely correlate with inhibition of NF-κB activation and reduction of NO formation (D'Acquisto *et al.*, (1999) Eur. J. Pharmacol. 369, 223-236; D'Acquisto *et al.*, (1999) Naunyn-Schmeideberg's Arch. Pharmacol. 360, 670-675). Therefore the wild-type NBD peptide is an effective inhibitor of inflammation in experimental animal models.

***Allowable Subject Matter***

4. Claims 3-13 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anand U. Desai, Ph.D. whose telephone number is (571) 272-0947. The examiner can normally be reached on Monday - Friday 7:00 a.m. - 3:30 p.m..

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jon P. Weber can be reached on (517) 272-0925. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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